



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>G01N 27/327, 27/333, 27/40, 33/53, 33/553, 33/569</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 97/44651</b> <b>(43) International Publication Date:</b> 27 November 1997 (27.11.97)
<b>(21) International Application Number:</b> PCT/AU97/00316 <b>(22) International Filing Date:</b> 22 May 1997 (22.05.97)  <b>(30) Priority Data:</b> 60/018,125                      22 May 1996 (22.05.96)                      US  <b>(71) Applicants (for all designated States except US):</b> AUSTRALIAN MEMBRANE AND BIOTECHNOLOGY RESEARCH INSTITUTE [AU/AU]; 126 Greville Street, Chatswood, NSW 2067 (AU). THE UNIVERSITY OF SYDNEY [AU/AU]; Sydney, NSW 2006 (AU).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> HARDING, Margaret [AU/AU]; 4/1-3 Percival Street, Lilyfield, NSW 2040 (AU). LUCAS, Sally, Wright [AU/AU]; 6/37 Carr Street, Coogee, NSW 2034 (AU). ARENSDORF, Patrick [US/US]; Apartment 1D, 327 West 85th Street, New York, NY 10024 (US). BRAACH-MAKSVYTIS, Vijoleta, Lucija, Bronislava [AU/AU]; 9 Darley Street, Dulwich Hill, NSW 2203 (AU).  <b>(74) Agent:</b> F.B. RICE & CO.; 28A Montague Street, Balmain, NSW 2041 (AU).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> NUCLEIC ACID SENSOR  <b>(57) Abstract</b>  The present invention provides biosensors and methods for the detection selected nucleic acid sequences. In one form the biosensor comprises an electrode and a bilayer membrane having a top and a bottom layer. The bottom layer being proximal to and connected to the electrode in a manner such that a space exists between the membrane and the electrode, with the conductance or impedance of the membrane being dependent on the presence or absence of the selected nucleic acid sequence. The membrane comprises a closely packed array of amphiphilic molecules and a plurality of ion channels comprising first half membrane spanning monomers dispersed in the top layer and second half membrane spanning monomers dispersed in the bottom layer. The first half membrane spanning monomers are capable of lateral diffusion within the upper layer and the second half membrane-spanning monomers are prevented from lateral diffusion within the bottom layer. A first ligand specifically reactive with the selected nucleic acid sequence is attached to an end of a proportion of the first half membrane-spanning monomers proximal the surface of the membrane and a second ligand reactive with the selected nucleic acid or marker attached thereto is attached to an end of the remainder of the first half membrane-spanning monomers proximal the surface of the membrane.		

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## ***Nucleic Acid Sensor***

### **FIELD OF INVENTION**

5 The present invention relates to a biosensor for use in detecting the presence of a selected nucleic acid sequence in a sample and to a method of detecting the presence of a selected nucleic acid (NA) sequence in a sample.

### **BACKGROUND OF THE INVENTION**

10 Assays for the presence of specific DNA or RNA sequences in samples have applications in many fields. For example, infection of patients with a particular microorganism can be assessed by analyzing a biological sample from the patient for the presence of a NA sequence specific for the microorganism. Other applications include the analysis of food or environmental samples to detect contamination. The detection of genetic disease caused by mutations can also be achieved by these techniques.

15 To date, the widespread use of this highly sensitive method has been restricted by the need for significant amplification of the amount of the specific nucleic acid sequence and/or the need to use specialized techniques to analyze the amplified DNA. This amplification is generally achieved using polymerase chain or ligase chain reaction and the amplified DNA is then separated on a gel and the gel analyzed for the presence of specific bands of DNA.

20 Other methods include US 4,840,893, which teaches a method of nucleic acid detection. The method uses a probe sequence with attached ligand, an enzyme mediator system linked to a second ligand and which is capable of transferring a charge to an electrical surface when the enzyme is catalytically active, and an antiligand, to which the first and second ligands compete for binding. The assay is a competition system whereby the binding of the target sequence in a sample affects the availability of the first ligand and alters the rate of charge transfer to the electrode. The present invention has the advantage of providing a direct gating mechanism, which does not rely on a signal generated by enzyme catalysis.

25 U.S. 4,868,104 describes a method of detecting a nucleotide sequence by the use of two polynucleotide reagents, which bind to different sequences on the target analyte, the first of which can be polymerized and the second has a detection system. Several other patents describe nucleic acid detection methods, most of which involve labelling of the probes. These

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include: U.S. 4,968,602; U.S. 5,116,733; U.S. 4,868,105; U.S. 4,716,106; U.S. 4,883,750; U.S. 5,242,794; U.S. 4,996,142; U.S. 5,348,855 and U.S. 5,053,326.

#### BRIEF DESCRIPTION OF DRAWINGS

- 5           Figures 1 to 3 show the results obtained in Examples 1 to 3.  
          Fig. 4 shows linker gramicidin B  
          Fig. 5 shows membrane spanning lipid  
          Fig. 6 shows linker lipid A  
          Fig. 7 shows biotinylated gramicidin E

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#### DESCRIPTION OF THE INVENTION

          The present inventors propose an apparatus which can be used to detect the presence of a specific NA sequence in a sample. The apparatus is best described as a biosensor in that it involves the use of lipid membranes. Membranes for the use in biosensors have been disclosed In international Patent Application Nos PCT/AU88/00273, PCT/AU89/00352 PCT/AU90/00025 and PCT/AU92/00132. The disclosure of these applications is included herein by reference.

15           As disclosed in these applications, suitably modified lipid molecules may be caused to assemble into an electrode/ionic reservoir/insulating bilayer combination that is suitable for incorporation of ion channels and ionophores. It is also disclosed that the conductance of these membranes is dependent on the presence or absence of an analyte. In bilayer membranes in which each layer includes ion channel monomers, the conductance of the membrane is dependent on the lining up of the monomers in each layer to form continuous ion channels which span the membrane. As these continuous ion channels are constantly being formed and destroyed, the conductance of the membrane is dependent on the lifetimes of these continuous ion channels.

20           In a first aspect, the present invention consists in a biosensor for use in detecting the presence of a selected nucleic acid sequence in a sample, the biosensor comprising an electrode and a bilayer membrane having a top and a bottom layer, the bottom layer being proximal to and connected to the electrode in a manner such that a space exists between the membrane and the electrode, the conductance or impedance of the membrane being dependent on the presence or absence of the selected nucleic acid sequence,

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the membrane comprising a closely packed array of amphiphilic molecules and a plurality of ion channels comprising first half membrane spanning monomers dispersed in the top layer and second half membrane spanning monomers dispersed in the bottom layer, the first half membrane spanning monomers being capable of lateral diffusion within the upper layer and the second half membrane-spanning monomers being prevented from lateral diffusion within the bottom layer, a first ligand specifically reactive with the selected nucleic acid sequence attached to an end of a proportion of the first half membrane-spanning monomers proximal the surface of the membrane and a second ligand reactive with the selected nucleic acid or marker attached thereto is attached to an end of the remainder of the first half membrane-spanning monomers proximal the surface of the membrane.

In a preferred embodiment of the present invention the membrane includes membrane spanning amphiphiles which are prevented from lateral diffusion within the membrane.

In a second aspect, the present invention consists in a biosensor for use in detecting the presence of a selected nucleic acid sequence in a sample, the biosensor comprising an electrode and a bilayer membrane having a top and a bottom layer, the bottom layer being proximal to and connected to the electrode in a manner such that a space exists between the membrane and the electrode, the conductance or impedance of the membrane being dependent on the presence or absence of the selected nucleic acid sequence, the membrane comprising a closely packed array of amphiphilic molecules, membrane spanning amphiphiles which are prevented from lateral diffusion within the membrane and a plurality of ion channels comprising first half membrane spanning monomers dispersed in the top layer and second half membrane spanning monomers dispersed in the bottom layer, the first half membrane spanning monomers being capable of lateral diffusion within the upper layer and the second half membrane-spanning monomers being prevented from lateral diffusion within the bottom layer, a first ligand specifically reactive with the selected nucleic acid sequence attached to either an end of at least a proportion of the first half membrane-spanning monomers proximal the surface of the membrane or to an end of the membrane-spanning amphiphiles proximal the surface of the membrane and a second ligand reactive with the selected nucleic acid or marker attached thereto attached to the other of the at least a proportion of the first half

membrane-spanning monomers proximal the surface of the membrane or the end of the membrane-spanning amphiphiles proximal the surface of the membrane.

5 In a third aspect the present invention consists in a method of detecting the presence of a selected nucleic acid sequence within a sample, the method comprising adding the sample to the biosensor of the first or second aspect of the present invention and detecting change in the impedance or conductance of the membrane.

10 As will be understood by those of moderate skill in the art the biosensor and method of the present invention functions by the binding of the selected nucleic acid sequence to the first and second ligands causing a change in the ability of ions to traverse the membrane via the ion channels. The specificity of the detection is provided by the specificity of the first  
15 ligand for the selected nucleic acid sequence. This specificity may be provided in a number of ways, however, it is presently preferred that this is achieved by the use of first ligand which comprises a nucleic acid molecule or PNA which includes a sequence complementary to a first sequence within the selected nucleic acid. As will be understood the sequence within the first  
20 ligand may be of any length sufficient to provide the required level of sensitivity, typically at least 10 residues. It will also be understood the nucleic acid molecule may include modified bases.

As the specificity is provided primarily by the first ligand it is not essential that the second ligand is specific for the selected nucleic acid sequence, it is simply necessary that the second ligand binds nucleic acid or  
25 a marker attached thereto. Accordingly, the second ligand may be an antibody directed against DNA, or binding fragment thereof. The second ligand may also be an intercalating agent, major or minor groove binder or an agent capable of triple helix formation or an agent capable of covalently  
30 linking to nucleic acids upon activation or combinations thereof. It is preferred that the second ligand is not specific for the selected nucleic acid sequence as this results in simpler assembly of the biosensor as only one specific ligand need be produced for each selected nucleic acid sequence that is to be detected.

35 The biosensor may also include a variety of second ligands which vary over the biosensor. For example the second ligand attached to one membrane spanning amphiphile may be a different moiety to the second



ligand attached to another membrane spanning amphiphile. The essential feature is that the second ligand binds to nucleic acid or to a marker attached thereto. Accordingly, it is to be understood that the term "second ligand" embraces a single or multiple species of moieties which bind nucleic acid or to a marker attached thereto.

As will be appreciated by those skilled in the field in a number of applications the nucleic acid sequence to be detected will be labelled with a marker, for example a biotinylated PCR product.

It is, however, also clearly within the ambit of the present invention that both the first and second ligands are specific for the selected nucleic acid sequence. In such an embodiment the first and second ligands are specific for different sequences within the selected nucleic acid sequence.

The first and second ligands may be attached in any of a number of ways. For example, streptavidin may be attached as described in the applications set out above. This can then be used to bind biotinylated nucleic acid. It is preferred that the first and second ligands are attached via linkers to the ion channels or membrane spanning lipids. Where a linker is used it is preferred that the linker is hydrophilic. It is further preferred that the linker includes a phosphoramidite group.

It is also expected that changing the linker length may affect the gating response detected in the biosensor.

Where the first and second ligands are nucleic acid sequences this may be advantageously achieved by producing a molecule having the two DNA sequences which are to act as the ligands at each end with an RNA sequence in the middle. The two ends of the molecule are then attached to the biosensor surface and the central RNA portion digested with RNaseH to leave the biosensor with the two separated DNA sequences attached.

In a preferred embodiment the first ligand comprises a nucleic acid molecule which includes a sequence complementary to a first sequence within the selected nucleic acid. It is further preferred that the second ligand comprises a nucleic acid molecule which includes a sequence complementary to a second sequence within the selected nucleic acid. Due to the presence of such complementary sequences the first and second ligands bind to the selected nucleic acid by hybridisation.

It is presently preferred that the first and second ligands are oligonucleotide sequences selected to bind to different regions of the selected

nucleic acid sequence in the sample. Methods to detect or sense specific NA sequences using the impedance bridge described in International Patent Application Nos PCT/AU88/00273, PCT/AU88/00273, PCT/AU89/00352, PCT/AU90/00025 and PCT/AU92/00132, may, for example, be achieved by  
5 manufacturing the membrane with both biotinylated membrane spanning lipids (MSL) and biotinylated gramicidin. Streptavidin, which binds strongly to biotin is immobilized by reaction with these biotinylated compounds.

A mixture of two biotinylated oligonucleotide sequences (first and second ligands) are then attached to the streptavidin. These sequences are preferably non complementary. One sequence would be a suitable probe for  
10 a sequence of interest e.g., an infectious organism probe sequence, and the other sequence would be the complement to another part of the target DNA.

Upon addition of the DNA to be detected, cross linking of the two biotinylated sequences occurs if the DNA contains the sequences which  
15 hybridise to the two attached sequences but not otherwise.

In a fourth aspect the present invention consists in a method of detecting the presence of a selected nucleic acid within a sample, the method comprising the following steps:

- (i) providing a biosensor comprising an electrode and a bilayer  
20 membrane having a top and a bottom layer, the bottom layer being proximal to and connected to the electrode in a manner such that a space exists between the membrane and the electrode, the conductance or impedance of the membrane being dependent on the presence or absence of the selected nucleic acid sequence, the  
25 membrane comprising a closely packed array of amphiphilic molecules and a plurality of ion channels comprising first half membrane spanning monomers dispersed in the top layer and second half membrane spanning monomers dispersed in the bottom layer, the first half membrane spanning monomers being capable of lateral  
30 diffusion within the upper layer and the second half membrane-spanning monomers being prevented from lateral diffusion within the bottom layer, a first ligand specifically reactive with a first nucleic acid sequence attached to an end of a proportion of the first half membrane-spanning monomers proximal the surface of the  
35 membrane and a second ligand specifically reactive with a second nucleic acid sequence attached to an end of the remainder of the first



half membrane-spanning monomers proximal the surface of the membrane, either the first or second nucleic acid sequence being within the sequence of a selected nucleic acid;

(ii) adding a sample suspected to contain the selected nucleic acid to the biosensor and measuring the conductance and/or impedance of the membrane; and

(iii-) adding a challenge nucleic acid to the biosensor from (ii) and measuring the conductance and/or impedance of the membrane, the challenge sequence being selected such that it includes both the first and second nucleic acid sequences.

If the target nucleic acid is present in the sample, the challenge sequence is unable to bind to both the first and second ligands and thus, crosslink. An absence of a difference in the conductance/impedance measurements in (ii) and (iii) being indicative of the presence of the selected nucleic acid sequence in the sample.

In a fifth aspect the present invention consists in a method of detecting the presence of a selected nucleic acid sequence within a sample, the method comprising the following steps:

(i) providing a biosensor comprising an electrode and a bilayer membrane having a top and a bottom layer, the bottom layer being proximal to and connected to the electrode in a manner such that a space exists between the membrane and the electrode, the conductance or impedance of the membrane being dependent on the presence or absence of the selected nucleic acid sequence, the membrane comprising a closely packed array of amphiphilic molecules, membrane spanning amphiphiles which are prevented from lateral diffusion within the membrane and a plurality of ion channels comprising first half membrane spanning monomers dispersed in the top layer and second half membrane spanning monomers dispersed in the bottom layer, the first half membrane spanning monomers being capable of lateral diffusion within the upper layer and the second half membrane-spanning monomers being prevented from lateral diffusion within the bottom layer, a first ligand specifically reactive with the selected nucleic acid sequence attached to either an end of at least a proportion of the first half membrane-spanning monomers proximal the surface of the

5 membrane or to an end of the membrane-spanning amphiphiles proximal the surface of the membrane and a second ligand specifically reactive with a second nucleic acid sequence attached to the other of the at least a proportion of the first half membrane-spanning monomers proximal the surface of the membrane or an end of the membrane-spanning amphiphiles proximal the surface of the membrane;

(ii) adding a sample suspected to contain the selected nucleic acid to the biosensor and measuring the conductance and/or impedance of the membrane; and

10 (iii-) adding a challenge nucleic acid to the biosensor from (ii) and measuring the conductance and/or impedance of the membrane, the challenge sequence being selected such that it includes both the first and second nucleic acid sequences.

15 If the target nucleic acid is present in the sample, the challenge sequence is unable to bind to both the first and second ligands and thus, crosslink. An absence of a difference in the conductance/impedance measurements in (ii) and (iii) being indicative of the presence of the selected nucleic acid sequence in the sample.

20 In a sixth aspect the present invention consists in a biosensor for use in detecting the presence of a selected nucleic acid sequence in a sample, the biosensor comprising an electrode and a bilayer membrane having a top and a bottom layer, the bottom layer being proximal to and connected to the electrode in a manner such that a space exists between the membrane and the electrode, the conductance or impedance of the membrane being  
25 dependent on the presence or absence of the selected nucleic acid sequence, the membrane comprising a closely packed array of amphiphilic molecules and a plurality of ion channels comprising first half membrane spanning monomers dispersed in the top layer and second half membrane spanning monomers dispersed in the bottom layer, the first half membrane spanning monomers being capable of lateral diffusion within the upper layer and the  
30 second half membrane-spanning monomers being prevented from lateral diffusion within the bottom layer, a first ligand specifically reactive with the selected nucleic acid sequence attached to an end of a proportion of the first half membrane-spanning monomers proximal the surface of the membrane  
35 and a second ligand attached to an end of the remainder of the first half

membrane-spanning monomers proximal the surface of the membrane, the second ligand having bound thereto or included therein the selected nucleic acid sequence such that the first ligands binds to the second ligand.

5 In a seventh aspect the present invention consists in a biosensor for use in detecting the presence of a selected nucleic acid sequence in a sample, the biosensor comprising an electrode and a bilayer membrane having a top and a bottom layer, the bottom layer being proximal to and connected to the electrode in a manner such that a space exists between the membrane and the electrode, the conductance or impedance of the membrane being  
10 dependent on the presence or absence of the selected nucleic acid sequence, the membrane comprising a closely packed array of amphiphilic molecules, membrane spanning amphiphiles which are prevented from lateral diffusion within the membrane and a plurality of ion channels comprising first half membrane spanning monomers dispersed in the top layer and second half  
15 membrane spanning monomers dispersed in the bottom layer, the first half membrane spanning monomers being capable of lateral diffusion within the upper layer and the second half membrane-spanning monomers being prevented from lateral diffusion within the bottom layer, a first ligand specifically reactive with the selected nucleic acid sequence attached to  
20 either an end of at least a proportion of the first half membrane-spanning monomers proximal the surface of the membrane or to an end of the membrane-spanning amphiphiles proximal the surface of the membrane and a second ligand attached to the other of the at least a proportion of the first half membrane-spanning monomers proximal the surface of the membrane or  
25 the end of the membrane-spanning amphiphiles proximal the surface of the membrane, the second ligand having bound thereto or included therein the selected nucleic acid sequence such that the first ligands binds to the second ligand.

30 In the arrangements specified in the sixth and seventh aspects the biosensor detects the presence of the selected nucleic acid sequence by competition. The selected nucleic acid sequence, if present in sample to be tested, competes with the second ligand for binding to the first ligand.

35 In an eighth aspect the present invention consists in a biosensor for use in detecting the presence of a selected nucleic acid sequence in a sample, the biosensor comprising an electrode and a bilayer membrane having a top and a bottom layer, the bottom layer being proximal to and connected to the

electrode in a manner such that a space exists between the membrane and the electrode, the conductance or impedance of the membrane being dependent on the presence or absence of the selected nucleic acid sequence, the membrane comprising a closely packed array of amphiphilic molecules and a plurality of ion channels comprising first half membrane spanning monomers dispersed in the top layer and second half membrane spanning monomers dispersed in the bottom layer, the first half membrane spanning monomers being capable of lateral diffusion within the upper layer and the second half membrane-spanning monomers being prevented from lateral diffusion within the bottom layer and a first ligand specifically reactive with the selected nucleic acid sequence attached to an end of a proportion of the first half membrane-spanning monomers proximal the surface of the membrane.

In this arrangement upon addition of sample containing the selected nucleic acid sequence the first ligand will bind to the selected DNA sequence and change the mobility of the first half membrane monomers or restrict ion flow through the channel. This will result in a membrane with a different conductance.

In another preferred embodiment of the invention, the first and second half membrane spanning monomers are gramicidin or one of its derivatives.

In a further preferred embodiment of the present invention, the bilayer membrane is attached to the electrode via linking molecules such that a space exists between the membrane and the electrode. Preferred linking molecules are those disclosed in application PCT/AU92/00132. In yet a further preferred embodiment of the present invention, the second half membrane spanning monomers are attached to the electrode via linker groups.

In yet another preferred embodiment of the present invention, the bilayer membrane includes membrane spanning lipids, similar to those found in archaebacteria.

"Cross linking" is characterized by the cross linking sequence bringing the first half membrane spanning monomers out of alignment with the second half membrane spanning monomers. This results in a membrane having a second conductance which is different from the first conductance.

This difference in conductance can be measured and will be indicative of the presence of the selected DNA sequence in the sample.

The first and second ligands may be the same or different.

5 Oligonucleotide ligands which bind to specific NA sequences are well known in the art.

In an alternative embodiment of the present invention the ligands are peptide nucleic acids (PNA). Information regarding PNA may be found in Science, 1991, 254, 1497 1500; J. Am. Chem. Soc., 1992, 114, 1895 1897, J. Am. Chem. Soc., 1992, 114, 9677 78; J. Chem. Soc. Chem. Commun., 10 1993, 800 801. Nature, 1993, 365, 566, 68; US Patent No. 4,795,700 and International Patent Application No. WO 93/18187 and the disclosure of these documents is included herein by reference. There are a number of advantages gained by the use of PNA as the ligands. These include the ability of PNA to recognize specific sequences in double stranded DNA and 15 to bind thereto either by strand displacement or triple helix formation. This is in contrast to oligonucleotides which can bind single strand or double strand but not by strand displacement. It should be noted that PNA can also bind to single stranded DNA. The target probe sequence could be a PNA molecule for tighter binding.

20 In an alternative embodiment, a mixture of two biotinylated oligonucleotide sequences are immobilized onto streptavidin. One is the target probe sequence and the second is any appropriate cross linking sequence of choice. On addition of sample, the target analyte is captured. The target analyte need only have the sequence of interest. No cross linking 25 occurs, therefore gating is not observed and a first measurement of conductance is taken. A second oligonucleotide sequence is then used to challenge the system. This challenge sequence contains both the target oligonucleotide sequence and the complementary sequence to the cross linking sequence. If binding of both epitopes occurs, cross linking will take 30 place, thus gating off the ion channels and changing the conductance of the membrane. If binding of only epitope two occurs (owing to the binding of the target DNA to its probe sequence) no cross linking takes place, thus no gating is seen. An absence of a change in conductance following addition of the challenge sequence is therefor an indication of the presence of the target 35 analyte.

The NA probe sequence, in principle, can be of any length and will contain the base sequence complementary to the target analyte for detection. The target analyte can be a DNA or an RNA sequence. A PNA probe sequence may also be of any length and will contain the base sequence complementary to the target analyte for detection. The target analyte can be a DNA or an RNA sequence as PNA binds to both. PNA binds DNA and RNA more tightly than does DNA, therefore the challenge sequence should not be able to displace the already bound target sequence.

Some examples of target sequences are listed below.

*Listeria monocytogenes*; a 19 base pair oligonucleotide that is specific for a 36S rRNA sequence; detection in food industry (milks, cheeses etc.) See: R. F. Wang W. W. Cao and M.G. Johnson, App. Environ. Microbiol., 1991, 3666 - 70.

All cholera toxin producing Strains of *Vibrio cholerea c* hybridise specifically with a 23 base pair oligonucleotide sequence (water detection, health). See: A C. Wright et al., J. Clin. Microbiol., 1992, 2302.

Specific oligonucleotide probes for detection of various strains of Chlamydia and Gonorrhoea (health industry). See: R. Rossau et al, u. Clin. Microbiol., 1990, 944 8; J. Gen. Microbiol, 1989, 135, 1735 45; Loeffelholz, M. J, J. Clin. Microbiol., 1992, 2847 51.

An excellent summary of current technologies that use DNA probes is found In "DNA Probes", 2nd Ed.: G. H Keller & M. M. Manak, Stockton Press, 1993.

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following non-limiting examples.

### Example 1: Lateral Segregation of DNA

Detection of target polynucleotide sequences can be illustrated by the following example in which specific DNA gating is observed for a target DNA concentration of 5 nM (1 pmole). The total lack of any response in the presence of a non-complementary probe sequence can be seen in the controls.



1st layer: 9.3nM Linker Gramicidin B  
1.1μM Membrane spanner Lipid D  
27.5nM Membrane spanner Lipid C  
5 37μM dithio diglyconic acid  
75μM Linker Lipid A  
2nd layer: 14mM (DPE-PC:GDPE=7:3) : Biotinylated Gramicidin E =  
50,000:1 in ethanol

10 Electrodes with freshly evaporated gold (1000Å) on chrome adhesion  
layer (200Å) on glass microscope slides) were dipped into an ethanolic  
solution of the first layer components for 1 hr at room temperature, rinsed  
with ethanol, then stored at 4°C under ethanol until used for impedance  
15 measurements. The slide was clamped into a block containing teflon coated  
wells which defined the area of the working electrode as approximately  
16mm<sup>2</sup>.

All steps were carried out at room temperature. 5μL of the 2nd layer  
was added to the working electrode before addition of a 180μL volume of  
phosphate buffered saline (10mM Na<sub>2</sub>HPO<sub>4</sub>, 1mM KH<sub>2</sub>PO<sub>4</sub>, 137mM NaCl,  
20 2.7mM KCl, PBS). The electrode was washed 4 times using PBS.  
Streptavidin was added to all wells (5μL 0.01mg/mL in PBS) and allowed to  
react with biotinylated Gramicidin E for 10-15 minutes before washing out  
excess unbound streptavidin with PBS. 5μL of a 1:1 mixture of DNA probe F  
(200nM):DNA probe G (200nM in PBS) was added to sensor wells. A DNA  
25 non-specific binding probe H (5μL 400 nM in PBS) was added to control  
wells. The probes were allowed to react with streptavidin for 10-15 minutes  
then excess unbound probes were washed out with PBS. 100μL of DNA  
target I (10nM) in PBS was added to each well. The binding of DNA target I  
to the sensor wells gave a decrease in the admittance at minimum phase, but  
30 no significant change in membrane admittance in control wells (Figure 1).  
The amount and rate of decrease of admittance at minimum phase is related  
to the amount of DNA present in the test solution and therefore can be used  
to determine concentration in test solutions.

## DNA probe F:

5'biotinylated listeria probe DNA with a 31-atom phosphoramidite linker group between the biotin and DNA.

5 5'-bio-L-M-ATAGTTTTATGGGATTAGC-3'

## DNA probe G:

5'biotinylated cholera toxin probe DNA with a 31-atom phosphoramidite linker group between the biotin and DNA.

10 5'-bio-L-M-CTCCGGAGCATAGAGCTTGGAGG-3'

## DNA non-specific binding probe H:

15 5'biotinylated 15-mer oligonucleotide with a 31-atom phosphoramidite linker group between the biotin and DNA, which is non-complementary to all parts of the target DNA sequence.

5'-bio-L-M-ATTGCTACGTATACG-3'

## 20 DNA target I:

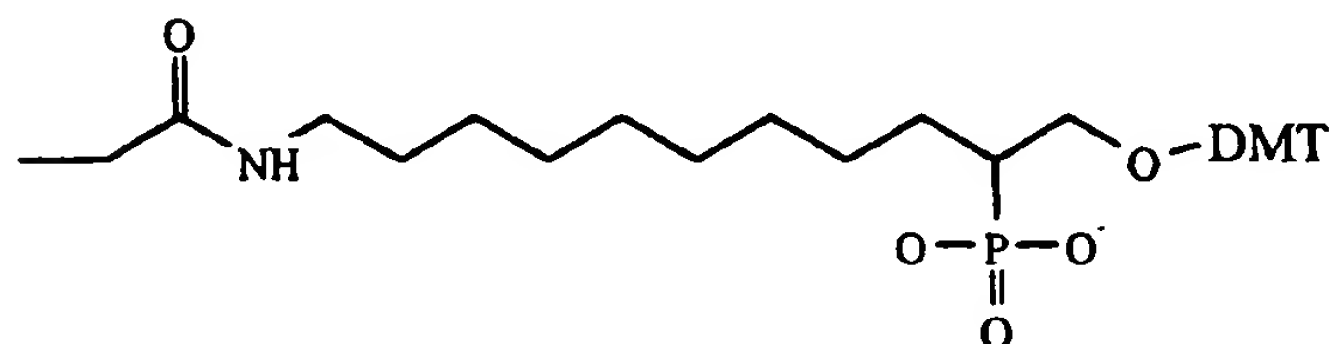
67 base DNA sequence containing the 19-base listeria sequence, a 10 base 'spacer' and the 23 base cholera toxin sequence.

25 5'-GCTAATCCCATAAAACTATGCATGCATGCATCGTACGTACGTACGTA  
CCCTCCAAGCTCTATGCTCCGGAG-3'

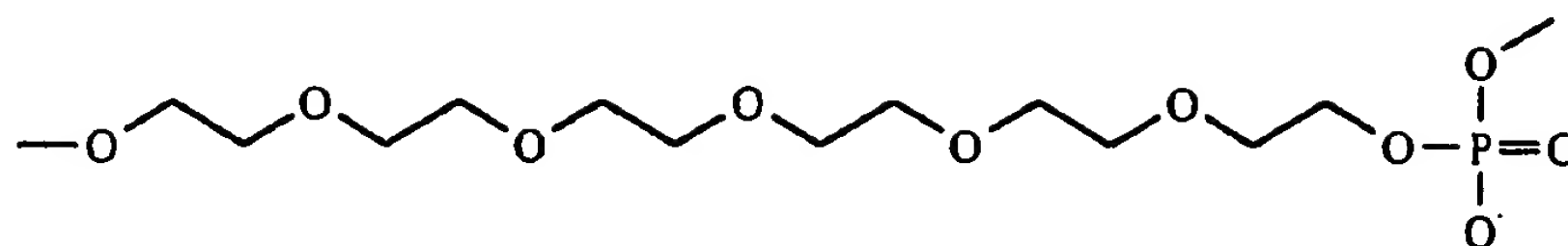
where:

bio = biotin

L =



M =



## 5 Example 2: Lateral Segregation of DNA followed by enzymatic cleavage

This example illustrates the ability to perform a second, confirmatory step whereby an enzyme can be used to cleave the bound polynucleotide, thus confirming that a specific region of interest is present.

10

1st layer: 9.3nM Linker Gramicidin B  
 1.1uM Membrane spanner Lipid D  
 27.5nM Membrane spanner Lipid C  
 37uM dithio diglyconic acid  
 75uM Linker Lipid A

15

2nd layer: 14mM (DPE-PC:GDPE=7:3) : Biotinylated Gramicidin E =  
 50,000:1 in ethanol

20

Electrodes were prepared and 2<sup>nd</sup> layer added as described in Example 1. Subsequent streptavidin, DNA probe and DNA target sequences were added as described in Example 1, except that they were carried out at 30°C.

25

After 15 minutes of DNA gating, unbound DNA target I was washed out with DNase I activation buffer. DNase I activation buffer consists of 50 nM Tris.HCl, pH 7.6, 50 nM NaCl, 10 nM MgCl<sub>2</sub>, 10 nM MnCl<sub>2</sub>, 0.2 mg/mL BSA. DNase I was added (2 µL 1mg/mL in a 50%<sub>w/v</sub> glycerol solution of 20 mM Tris.HCl, pH 7.6, 1 mM MgCl<sub>2</sub>) to sensor and control wells. Addition of DNase I gave an increase in admittance at minimum phase for sensor wells, but no significant increase for control wells (figure 2), showing that cleavage of DNA was being detected.

30

**Example 3: DNA gating by addition of 2 biotinylated complementary polynucleotide sequences**

5 This example illustrates the possibility of detecting only one specific sequence on the polynucleotide of interest by having a capture molecule incorporated in the target. It also illustrates the use of peptide nucleic acid (PNA) sequences.

10 1st layer: 9.3nM Linker Gramicidin B  
1.1uM Membrane spanner Lipid D  
5.5nM Membrane spanner Lipid C  
37uM dithio diglyconic acid  
75uM Linker Lipid A  
2nd layer: 14mM (DPE-PC:GDPE=7:3) : Biotinylated Gramicidin E =  
15 50,000:1 in ethanol

20 Electrodes were prepared and 2<sup>nd</sup> layer added as described in Example 1. All steps were carried out at room temperature. 5µL of the 2nd layer was added to the working electrode before addition of a 180µL volume of phosphate buffered saline (10mM Na<sub>2</sub>HPO<sub>4</sub>, 1mM KH<sub>2</sub>PO<sub>4</sub>, 137mM NaCl, 2.7mM KCl, PBS). The electrode was washed 4 times using PBS. Streptavidin was added to all wells (5µL 0.01mg/mL in PBS) and allowed to react with biotinylated Gramicidin E for 10-15 minutes before washing out excess unbound streptavidin with PBS.

25 5 nM of each of PNA sequence J and the complementary DNA sequence K (1.14µL 1.75 µM) were added quickly in succession to the sensor wells. No PNA or DNA sequence was added to the control wells. The binding of the PNA (J) and DNA (K) sequences to the streptavidin and each other gave a decrease in the admittance at minimum phase, but no  
30 significant change in membrane admittance in control wells (Figure 3). The amount and rate of decrease of admittance at minimum phase is related to the amount of DNA or PNA present in the test solution and therefore can be used to determine concentration in test solutions.

PNA sequence J:

N-biotinylated listeria probe sequence PNA containing glycine and lysine for solubility plus an 8-amino-3,6-dioxaoctanoic acid linker.

5 N-bio-gly-lys-O-ATAGTTTTATGGGATTAGC-CONH<sub>2</sub>

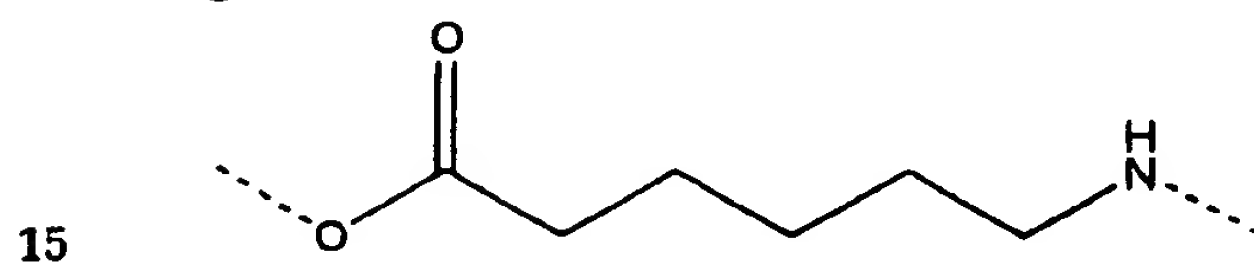
DNA sequence K:

5'-biotinylated listeria DNA with no linker group between the biotin and DNA.

10 5'-bio-GCTAATCCCATAAACTAT-3'

where

O =



It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. These include the detection of PCR (or other amplification method) products and the detection of genetic mutations in genetically derived diseases, where the mutation is known. A list of some 3500 conditions due to defective genes can be found in McKusick's "Mendelian Inheritance in Man" and further examples are regularly reported in the scientific press. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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## CLAIMS:-

1. A biosensor for use in detecting the presence of a selected nucleic acid sequence in a sample, the biosensor comprising an electrode and a bilayer membrane having a top and a bottom layer, the bottom layer being proximal to and connected to the electrode in a manner such that a space exists between the membrane and the electrode, the conductance or impedance of the membrane being dependent on the presence or absence of the selected nucleic acid sequence, the membrane comprising a closely packed array of amphiphilic molecules and a plurality of ion channels comprising first half membrane spanning monomers dispersed in the top layer and second half membrane spanning monomers dispersed in the bottom layer, the first half membrane spanning monomers being capable of lateral diffusion within the upper layer and the second half membrane-spanning monomers being prevented from lateral diffusion within the bottom layer, a first ligand specifically reactive with the selected nucleic acid sequence attached to an end of a proportion of the first half membrane-spanning monomers proximal the surface of the membrane and a second ligand reactive with the selected nucleic acid or marker attached thereto is attached to an end of the remainder of the first half membrane-spanning monomers proximal the surface of the membrane.
2. A biosensor for use in detecting the presence of a selected nucleic acid sequence in a sample, the biosensor comprising an electrode and a bilayer membrane having a top and a bottom layer, the bottom layer being proximal to and connected to the electrode in a manner such that a space exists between the membrane and the electrode, the conductance or impedance of the membrane being dependent on the presence or absence of the selected nucleic acid sequence, the membrane comprising a closely packed array of amphiphilic molecules and a plurality of ion channels comprising first half membrane spanning monomers dispersed in the top layer and second half membrane spanning monomers dispersed in the bottom layer, the first half membrane spanning monomers being capable of lateral diffusion within the upper layer and the second half membrane-spanning monomers being prevented from lateral diffusion within the bottom layer, a first ligand specifically reactive with the selected nucleic acid sequence attached to an end of a proportion of the first half membrane-



spanning monomers proximal the surface of the membrane and a second ligand attached to an end of the remainder of the first half membrane-spanning monomers proximal the surface of the membrane, the second ligand having bound thereto or included therein the selected nucleic acid sequence such that the first ligands binds to the second ligand.

5

3. A biosensor as claimed in claim 1 or claim 2 in which the membrane includes membrane spanning amphiphiles which are prevented from lateral diffusion within the membrane.

10

4. A biosensor for use in detecting the presence of a selected nucleic acid sequence in a sample, the biosensor comprising an electrode and a bilayer membrane having a top and a bottom layer, the bottom layer being proximal to and connected to the electrode in a manner such that a space exists between the membrane and the electrode, the conductance or impedance of the membrane being dependent on the presence or absence or the selected nucleic acid sequence, the membrane comprising a closely packed array of amphiphilic molecules, membrane spanning amphiphiles which are prevented from lateral diffusion within the membrane and a plurality of ion channels comprising first half membrane spanning monomers dispersed in the top layer and second half membrane spanning monomers dispersed in the bottom layer, the first half membrane spanning monomers being capable of lateral diffusion within the upper layer and the second half membrane-spanning monomers being prevented from lateral diffusion within the bottom layer, a first ligand specifically reactive with the selected nucleic acid sequence attached to either an end of at least a proportion of the first half membrane-spanning monomers proximal the surface of the membrane or to an end of the membrane-spanning amphiphiles proximal the surface of the membrane and a second ligand reactive with the selected nucleic acid or marker attached thereto attached to the other of the at least a proportion of the first half membrane-spanning monomers proximal the surface of the membrane or the end of the membrane-spanning amphiphiles proximal the surface of the membrane.

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5. A biosensor for use in detecting the presence of a selected nucleic acid sequence in a sample, the biosensor comprising an electrode and a bilayer membrane having a top and a bottom layer, the bottom layer being proximal to and connected to the electrode in a manner such that a space exists between the membrane and the electrode, the conductance or

- impedance of the membrane being dependent on the presence or absence of the selected nucleic acid sequence, the membrane comprising a closely packed array of amphiphilic molecules, membrane spanning amphiphiles which are prevented from lateral diffusion within the membrane and a plurality of ion channels comprising first half membrane spanning monomers dispersed in the top layer and second half membrane spanning monomers dispersed in the bottom layer, the first half membrane spanning monomers being capable of lateral diffusion within the upper layer and the second half membrane-spanning monomers being prevented from lateral diffusion within the bottom layer, a first ligand specifically reactive with the selected nucleic acid sequence attached to either an end of at least a proportion of the first half membrane-spanning monomers proximal the surface of the membrane or to an end of the membrane-spanning amphiphiles proximal the surface of the membrane and a second ligand attached to the other of the at least a proportion of the first half membrane-spanning monomers proximal the surface of the membrane or the end of the membrane-spanning amphiphiles proximal the surface of the membrane, the second ligand having bound thereto or included therein the selected nucleic acid sequence such that the first ligands binds to the second ligand.
- 5 6. A biosensor as claimed in any one of claims 1 to 5 in which the first ligand comprises a nucleic acid molecule or PNA which includes a sequence complementary to a first sequence within the selected nucleic acid.
- 10 7. A biosensor as claimed in any one of claims 1 to 6 in which the second ligand is an antibody directed against DNA, or binding fragment thereof.
- 15 8. A biosensor as claimed in any one of claims 1 to 6 in which the second ligand is an intercalating agent, major or minor groove binder, an agent capable of triple helix formation or an agent which can covalently couple to DNA.
- 20 9. A biosensor as claimed in any one of claims 1 to 6 in which the second ligand is specific for the selected nucleic acid sequence
- 25 10. A biosensor as claimed in any one of claims 1 to 9 in which the first and second ligands are attached via linkers to the ion channels or membrane spanning lipids.
- 30 11. A biosensor as claimed in claim 10 in which the linker is a phosphoramidite linker group.
- 35

12. A biosensor for use in detecting the presence of a selected nucleic acid sequence in a sample, the biosensor comprising an electrode and a bilayer membrane having a top and a bottom layer, the bottom layer being proximal to and connected to the electrode in a manner such that a space exists between the membrane and the electrode, the conductance or impedance of the membrane being dependent on the presence or absence of the selected nucleic acid sequence, the membrane comprising a closely packed array of amphiphilic molecules and a plurality of ion channels comprising first half membrane spanning monomers dispersed in the top layer and second half membrane spanning monomers dispersed in the bottom layer, the first half membrane spanning monomers being capable of lateral diffusion within the upper layer and the second half membrane-spanning monomers being prevented from lateral diffusion within the bottom layer and a first ligand specifically reactive with the selected nucleic acid sequence attached to an end of a proportion of the first half membrane-spanning monomers proximal the surface of the membrane.
13. A biosensor as claimed in claim 12 in which the first ligand comprises a nucleic acid molecule or PNA which includes a sequence complementary to a first sequence within the selected nucleic acid.
14. A biosensor as claimed in claim 12 or 13 in which the first ligand is attached via linkers to the ion channels.
15. A biosensor as claimed in claim 14 in which the linker is a phosphoramidite linker group.
16. A biosensor as claimed in any one of claims 1 to 15 in which the first and second half membrane spanning monomers are gramicidin or one of its derivatives.
17. A method of detecting the presence of a selected nucleic acid within a sample, the method comprising the following steps:
- (i) providing a biosensor comprising an electrode and a bilayer membrane having a top and a bottom layer, the bottom layer being proximal to and connected to the electrode in a manner such that a space exists between the membrane and the electrode, the conductance or impedance of the membrane being dependent on the presence or absence of the selected nucleic acid sequence, the membrane comprising a closely packed array of amphiphilic molecules and a plurality of ion channels comprising first half

- 5 membrane spanning monomers dispersed in the top layer and second  
half membrane spanning monomers dispersed in the bottom layer,  
the first half membrane spanning monomers being capable of lateral  
diffusion within the upper layer and the second half membrane-  
spanning monomers being prevented from lateral diffusion within the  
bottom layer, a first ligand specifically reactive with a first nucleic  
acid sequence attached to an end of a proportion of the first half  
membrane-spanning monomers proximal the surface of the  
membrane and a second ligand specifically reactive with a second  
10 nucleic acid sequence attached to an end of the remainder of the first  
half membrane-spanning monomers proximal the surface of the  
membrane, either the first or second nucleic acid sequence being  
within the sequence of a selected nucleic acid;
- (ii) adding a sample suspected to contain the selected nucleic acid to the  
15 biosensor and measuring the conductance and/or impedance of the  
membrane; and
- (iii-) adding a challenge nucleic acid to the biosensor from (ii) and  
measuring the conductance and/or impedance of the membrane, the  
challenge sequence being selected such that it includes both the first  
20 and second nucleic acid sequences.
18. A method of detecting the presence of a selected nucleic acid  
sequence within a sample, the method comprising the following steps:
- (i) providing a biosensor comprising an electrode and a bilayer  
25 membrane having a top and a bottom layer, the bottom layer being  
proximal to and connected to the electrode in a manner such that a  
space exists between the membrane and the electrode, the  
conductance or impedance of the membrane being dependent on the  
presence or absence of the selected nucleic acid sequence, the  
membrane comprising a closely packed array of amphiphilic  
30 molecules, membrane spanning amphiphiles which are prevented  
from lateral diffusion within the membrane and a plurality of ion  
channels comprising first half membrane spanning monomers  
dispersed in the top layer and second half membrane spanning  
monomers dispersed in the bottom layer, the first half membrane  
spanning monomers being capable of lateral diffusion within the  
35 upper layer and the second half membrane-spanning monomers being

- 5 prevented from lateral diffusion within the bottom layer, a first ligand specifically reactive with the selected nucleic acid sequence attached to either an end of at least a proportion of the first half membrane-spanning monomers proximal the surface of the membrane or to an end of the membrane-spanning amphiphiles proximal the surface of the membrane and a second ligand specifically reactive with a second nucleic acid sequence attached to the other of the at least a proportion of the first half membrane-spanning monomers proximal the surface of the membrane or an end of the membrane-spanning amphiphiles proximal the surface of the membrane;
- 10 (ii) adding a sample suspected to contain the selected nucleic acid to the biosensor and measuring the conductance and/or impedance of the membrane; and
- 15 (iii-) adding a challenge nucleic acid to the biosensor from (ii) and measuring the conductance and/or impedance of the membrane, the challenge sequence being selected such that it includes both the first and second nucleic acid sequences.
19. A method as claimed in claims 17 or 18 in which the first ligand comprises a nucleic acid molecule or PNA which includes a sequence complementary to a first sequence within the selected nucleic acid.
20. A method as claimed in any one of claims 17 to 19 in which the second ligand is an antibody directed against DNA, or binding fragment thereof.
- 25 21. A method as claimed in any one of claims 17 to 19 in which the second ligand is an intercalating agent, major or minor groove binder, an agent capable of triple helix formation or an agent which can covalently couple to DNA.
22. A method as claimed in any one of claims 17 to 19 in which the second ligand is specific for the selected nucleic acid sequence
- 30 23. A method as claimed in any one of claims 17 to 23 in which the first and second ligands are attached via linkers to the ion channels or membrane spanning lipids.
24. A method as claimed in claim 23 in which the linker is a phosphoramidite linker group.
- 35

25. A method as claimed in any one of claims 17 to 24 in which the first and second half membrane spanning monomers are gramicidin or one of its derivatives.



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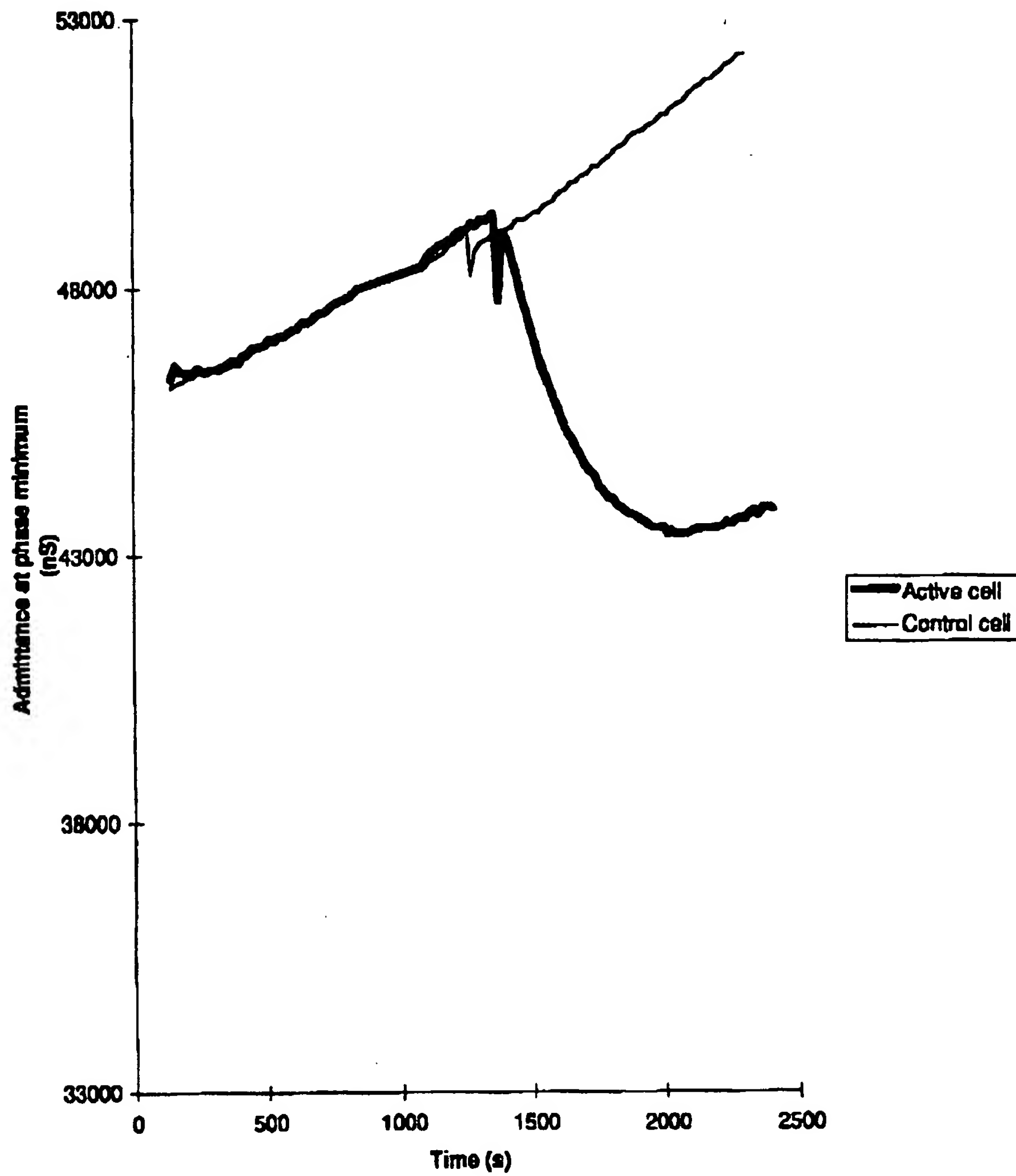


FIGURE 1

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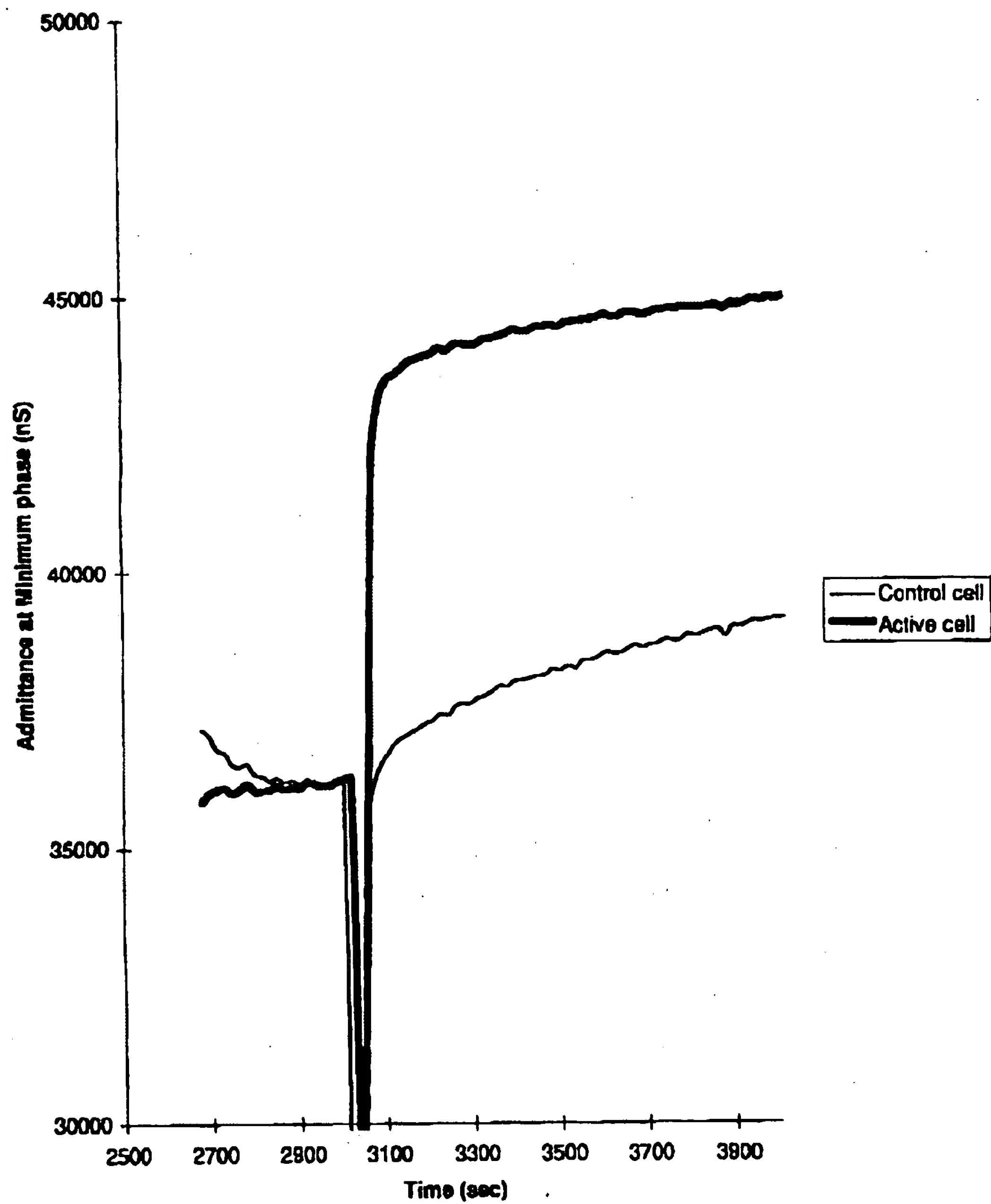


FIGURE 2

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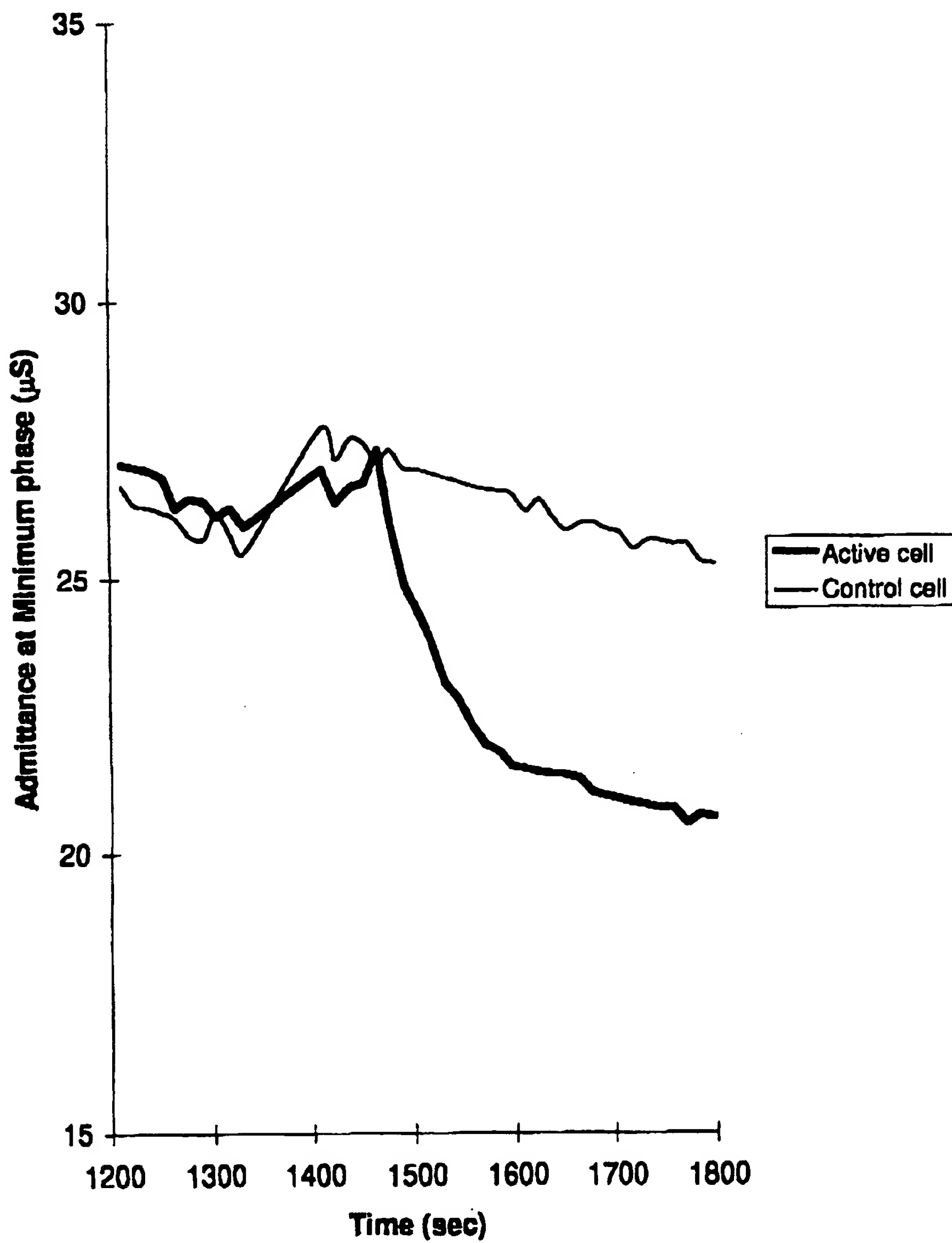


FIGURE 3



5/5

Figure 5: Membrane spanning lipid

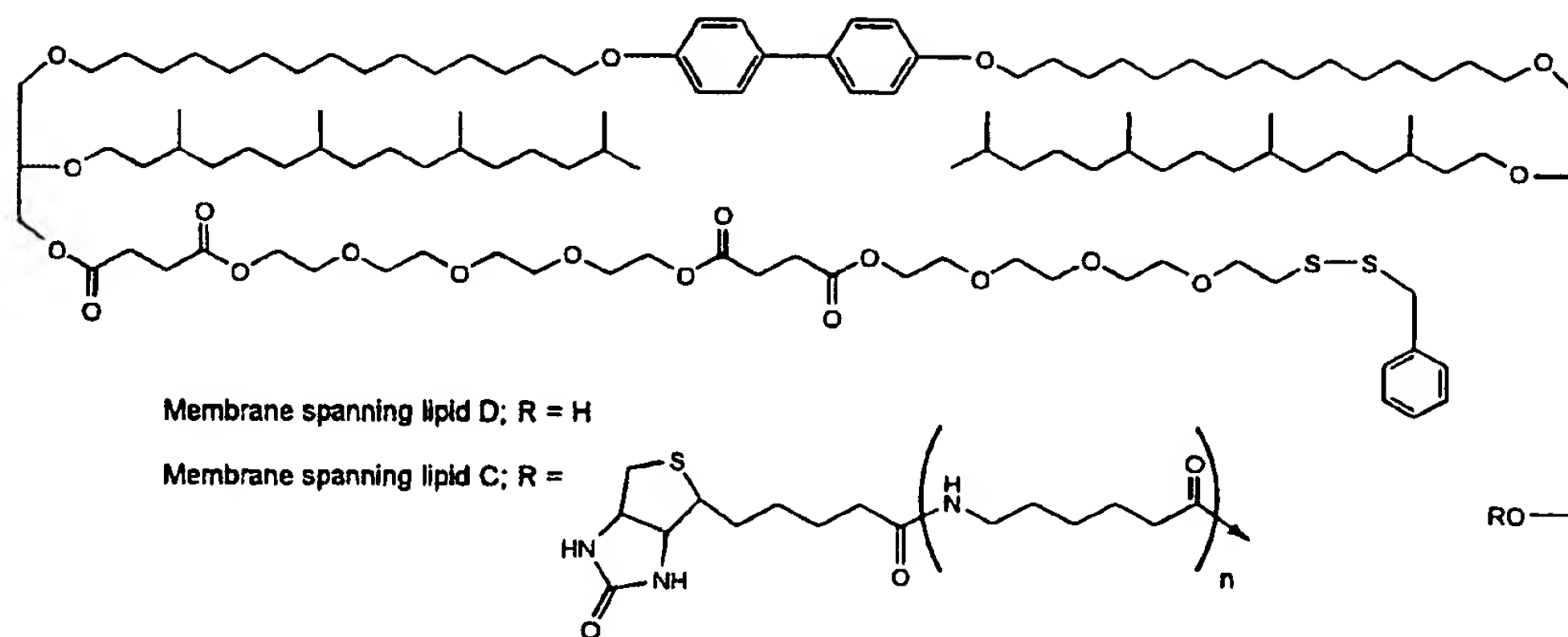


Figure 6: Linker lipid A

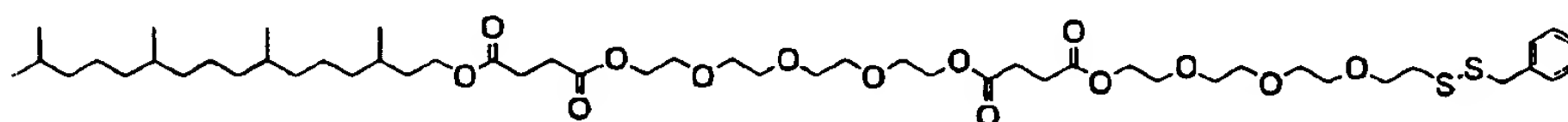
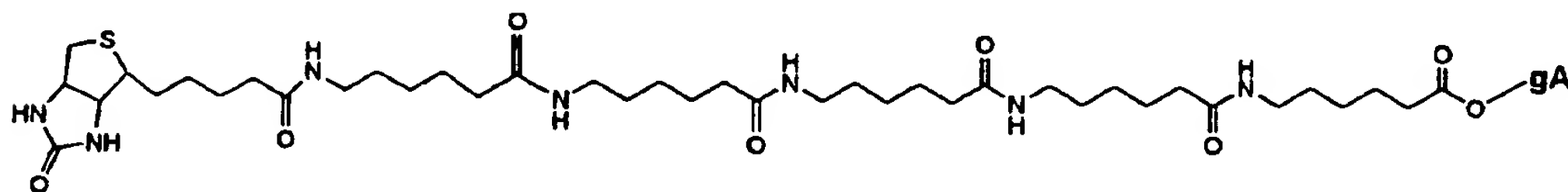


Figure 7: Biotinylated gramicidin E



where gA is as shown in Figure 4.

# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/AU 97/00316

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
Int Cl <sup>6</sup> : G01N 27/327, 27/333, 27/40, 33/53, 33/553, 33/569		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols) G01N 27/-, 33/-		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched AU: IPC G01N 37/327, 27/333, 27/40, 33/53, 33/553, 33/569		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) CHEMICAL ABSTRACTS, DERWENT (KEYWORDS: BIOSENSOR, ION CHANNEL, IONOPHORE, DNA, RNA, NUCLEIC ACID)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 97/01092 (Australian Membrane and Biotechnology Research Institute) 9 January 1997 (see entire document, in particular page 8 lines 14-15, claim 50)	1, 3-4, 6-10, 16
Y	WO 93/10212 (Case, G.D.; Worley J.F.) 27 May 1993 (see entire document, in particular page 8 lines 23-24, page 11 lines 29-35, page 14 lines 27-28, page 18 line 32 - page 19 line 11, example 13)	1-16
Y	WO 92/17788 (Australian Membrane and Biotechnology Research Institute) 15 October 1992 (see entire document)	1-16
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 13 August 1997		Date of mailing of the international search report <b>15 AUG 1997</b>
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929		Authorized officer  <b>NORMAN BLOM</b> Telephone No.: (06) 283 2238



# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/AU 97/00316

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 94/24562 (Australian Membrane and Biotechnology Research Institute and The University of Sydney) 27 October 1994 (see entire document, in particular page 1 lines 23-36, page 3 lines 9-11 and page 6 lines 4-10)	1-16
P, Y	WO 96/15454 (Australian Membrane and Biotechnology Research Institute and The University of Sydney) 23 May 1996 (see page 2 line 18 - page 3 line 20, page 4 lines 15-27, page 8 lines 7-16, page 10 lines 16-21 and figures 7a and 7b)	1-16
A	American Chemical Society, Symposium series (1994), 556 (Diagnostic Biosensor Polymers), 238-251. Maeda et al. "Semisynthetic Macromolecular Conjugates for Biomimetic Sensors" (see page 245-250, DNA-Binding Drug Sensor)	8, 12, 21
A	Chemical Abstracts, CAPLUS Online Abstracts, Abstract Number 217074 (1996), "New detector strategies for capillary-format separations" R.N. Zare, Book of Abstracts, 211th ACS National Meeting, New Orleans, LA March 24-28 (1996) (see entire abstract)	1

# INTERNATIONAL SEARCH REPORT

## Information on patent family members

International Application No.

**PCT/AU 97/00316**

**This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.**

<b>Patent Document Cited in Search Report</b>	<b>Patent Family Member</b>					
<b>WO     97/01092</b>	<b>AU     59926/96</b>					
<b>WO     93/10212</b>	<b>AU     31771/93</b>	<b>EP       643764</b>	<b>US       5328847</b>			
<b>WO     92/17788</b>	<b>AU     14657/92</b>	<b>CA       2106966</b>	<b>EP       639269</b>			
	<b>US     5401378</b>					
<b>WO     94/24562</b>	<b>AU     65327/94</b>	<b>CA       2161084</b>	<b>EP       695425</b>			
<b>WO     96/15454</b>	<b>AU     38643/95</b>					
<b>END OF ANNEX</b>						